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Experimental and Theoretical Analysis of Phase Equilibria in a Two-phase System Used for Biocatalytic Esterifications

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The partitioning behavior of the reactants 1-butanol, propionic acid and butyl propionate in an aqueous–organic two-phase system consisting of alginate beads suspended in hexane was investigated. Partitioning experiments with a single reactant showed that, even in the dilute region, the equilibrium concentrations of 1-butanol and propionic acid cannot be described by constant partition coefficients as is normally done in the field of biocatalysis. Besides the aqueous alginate beads, two other aqueous phases with different compositions (solutions with and without electrolytes) were also used for partitioning experiments. The equilibrium concentrations of the reactants obtained from the systems with the three different aqueous phases (water, water plus electrolytes, alginate beads) demonstrated that the partitioning behavior of the reactants is scarcely influenced by the presence of the electrolytes or by the alginate matrix, at least up to reactant concentrations of 80 mmol/l in the organic phase. The comparison of the experimental equilibrium concentrations with predicted values obtained from simulations with the modified UNIFAC (Dortmund) model showed a generally good agreement. However, in the dilute region, differences of up to 100% occurred between experimental and predicted values. Thus, for the later detailed mathematical modeling of processes occurring inside the alginate beads (such as mass transfer and enzymatic reaction), the modified UNIFAC (Dortmund) model is not adequate. Therefore, empirical correlations were derived for the mathematical description of the reactants' partitioning behavior. Experiments, conducted with two reactants simultaneously present in the two-phase system, showed that at reactant concentrations in the organic phase higher than 10 mmol/l the partitioning behavior of the investigated reactants is influenced by the presence of the second component. Thus, in systems with multiple reactants the derived correlations are strictly only valid up to this concentration.

Keywords: Enzymatic esterification; Alginate bead; Two-phase system; Phase equilibrium; UNIFAC

INTRODUCTION

Aqueous–organic two-phase systems are an attractive technique for enzymatic reactions involving hydrophobic products (for example, esterifications). The components present in the two-phase system (reactants and solvents) distribute themselves between the two phases until thermodynamic phase equilibrium is reached. For comparison of different types of two-phase systems, for assessment of the influence of organic solvents on the real intrinsic enzyme kinetics, and also for modeling purposes it is necessary to know the partitioning behavior of the reactants. Only with this information, can the actual concentrations of the reactants in the aqueous microenvironment of the enzyme be known. Without considering this thermodynamic effect, it is impossible to study the direct effects of an organic solvent on the properties of enzymes (Kasche *et al*., 1991).

In the field of biocatalysis with aqueous–organic two-phase systems, constant partition coefficients, representing the ratio of the reactant concentration in the organic phase to that in the aqueous phase, are most frequently employed to characterize the partitioning behavior of reactants (Antczak *et al*., 2001; Dias *et al*., 1991; Ferreira-Dias and da Fonseca, 1995; Frense *et al*., 1996; Monot *et al*., 1991; Mustranta *et al*., 1993; Suye *et al*., 2002; Wehtje and Adlercreutz, 1997; Wu *et al*., 2002; Yang and Robb, 1994). However, Eggers *et al.* (1989) stated that only in very dilute systems can partition coefficients be assumed to be constant while in non-ideal solutions the partition coefficients are a function of concentration.

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Therefore, Halling (1990) suggested that the predictive group contribution method UNIFAC be applied in the field of biocatalysis to describe phase equilibria. Thus, the original UNIFAC model (Fredenslund *et al*., 1975) with the parameters of Magnussen *et al.* (1981) was used to calculate partition coefficients (Wescott and Klibanov, 1993a,b), to study the effects of solvents on the enantioselectivity of a lipase (Parida and Dordick, 1993), to predict product concentrations at the equilibrium of an esterification reaction in a non-dilute system (Janssen *et al*., 1993a,b,c; van der Padt *et al*., 1993), to determine activity-based kinetic constants of enzymes (Janssen *et al*., 1996, 1999; Lee, 1995; van Tol *et al*., 1995a,b; Wehtje and Adlercreutz, 1997) and to investigate enzyme hydration in organic solvents (Lee and Kim, 1995). However, differences between experimentally determined and predicted activity coefficients were reported in most of these papers. This might be due to the fact that the original UNIFAC model cannot reliably predict the behavior in the dilute region particularly if systems with molecules of significantly different size are considered (Lohmann *et al*., 2001; Weidlich and Gmehling, 1987). Although improved UNIFAC methods exist and were also applied in the field of biocatalysis (Luque *et al*., 1998; Voutsas *et al*., 2002), the outdated original UNIFAC model (Fredenslund *et al*., 1975) with parameters of Magnussen *et al.* (1981) has still been used in recent publications which investigated equilibrium shifts caused by use of different organic solvents (Bellot *et al*., 2001; Flores *et al*., 2000).

In the cited biocatalysis papers dealing with UNIFAC models, only systems with trapped aqueous phases have been investigated. In these systems, the aqueous phase is very small and not always clearly visible, since it may be restricted to the pores of the suspended enzyme particles. Thus, no experimental validation of the theoretically obtained equilibrium concentrations has been attempted so far. Therefore, a two-phase biocatalysis system (Hertzberg *et al*., 1990, 1992), consisting of alginate beads suspended in hexane, has been investigated in this work. This system has a macroscopic large and stabilized aqueous phase. Thus, equilibrium concentrations of the reactants can be experimentally assessed allowing a comparison with corresponding UNIFAC predictions. This system, representing a model for various two-phase systems used for biotransformation, also allows investigation of the effect that biological buffers and other electrolytes, present in the aqueous phase in the vicinity of the enzyme, have on the reactant partitioning behavior.

Within this work, the partitioning behavior of 1-butanol, propionic acid and butyl propionate (representing typical reactants of an enzymatically (e.g. lipase) catalyzed esterification) was experimentally determined in different aqueous–organic two-phase systems and the results were subsequently compared with theoretical data. For this purpose, the latest UNIFAC model, namely the modified UNIFAC (Dortmund) model (Gmehling *et al*., 1993; Weidlich and Gmehling, 1987), was used.

MATERIALS AND METHODS

Chemicals

1-Butanol (99.5%), propionic acid (99%), and *n*-hexane (99%) were obtained from Merck (Germany). Butyl propionate (98%) was from Merck-Schuchardt (Germany). Decane (98%), used as internal standard for the gas chromatographic analysis, was purchased from Fluka (Switzerland).

For the production of alginate beads, Manugel® DJX sodium alginate was obtained from Monsanto (UK), and the biological buffer Tris(hydroxymethyl)-amino-methane (TRIS) (99%) was purchased from Fluka (Switzerland) and HCl (technical grade) from Roth (Germany). $CaCl_2 \cdot 2H_2O$ (99.5%) was obtained from Applichem (Germany). All chemicals were used without further purification.

Experimental Determination of Phase Equilibria

Experiments were conducted to determine the partitioning behavior of 1-butanol, propionic acid and butyl propionate between an aqueous solution and the organic solvent hexane. Mainly ternary systems with a single reactant present in the two solvent phases were studied. Additionally, some partitioning experiments were performed with a quaternary system where the reactants 1-butanol and propionic acid were present in the aqueous–organic two-phase system at the same time. For the partitioning experiments, a certain volume of a hexane solution containing an exactly defined amount of the considered reactant was added to a known volume of the aqueous phase. In order to characterize the influence of the alginate matrix and the electrolytes TRIS, HCl and $CaCl_2$, which are present inside the alginate beads, the partitioning experiments were performed with different aqueous solutions.

The three different aqueous solutions were prepared as follows: The first aqueous system (A) only consisted of fully deionized water whereas the second system (B) was composed of a solution of fully deionized water with 0.18 mol/l $CaCl_2$ and 0.1 mol/l TRIS, titrated with HCl (1 mol/l) to pH 7.5. The third aqueous system (C) consisted of alginate beads. For the preparation of the beads, 100 ml of a buffered (0.1 mol/l TRIS–HCl, pH 7.5) 2% (w/v) alginate solution were dropped in a 900 ml hardening bath consisting of an equally buffered 0.18 mol/l $CaCl_2$ solution. A syringe was used to obtain

beads with diameter between 3.3 and 3.6 mm. After 12 hours in the hardening bath, the beads were removed with a filter, briefly dried with a paper towel and stored in a water-saturated hexane solution until being used in the experiments.

The preparation of the organic phase solutions was the same for all experiments with the three different aqueous phases. Hexane solutions with different reactant concentrations (1–250 mmol/l) were prepared. For the experiments with the quaternary systems, solutions with equimolar concentrations of the two reactants were employed. The organic phase solutions also contained 20 mmol/l decane which was used as an internal standard for the gas chromatographic analysis. Since our UNIFAC calculations revealed that the partitioning of decane into the aqueous phase is almost non-existent under the conditions investigated here, decane could be used as an internal standard.

For the experiments, the organic phase solution was combined with the respective aqueous solution with a volumetric ratio between the organic and the aqueous phase that produced a significant decrease of the reactant concentration in the organic phase (volumetric ratio in experiments with propionic acid/1-butanol: 12.5; with butyl propionate: 0.07 and 0.2). The volume of the alginate bead was determined by dividing the weight of the alginate beads by their density. A density of 1.022 mg/ml had been determined in preceding experiments.

All the experiments were performed at a temperature between 291 and 294 K. The experiments with the fluid aqueous phases (systems A and B) were conducted in sealed glass bottles (50 ml), and those with alginate beads (system C) were carried out in GC vials (1.5 ml). Each vial was filled with three single beads and the appropriate volume of the organic phase. Due to very low equilibrium concentrations of the ester in the water phase, ester partitioning experiments with system C were not feasible. Therefore, the ester partitioning experiments were only conducted with systems A and B. In all experiments, the two-phase systems were allowed to equilibrate for 1 week. In preliminary experiments, it was verified that the equilibration of the phases is reached without stirring within this period of time. Additional preliminary experiments with the quaternary system demonstrated, that the reaction rate of the acid-catalyzed esterification is extremely low. Thus, the ester produced during the 1-week equilibration process could be neglected. In the ester partitioning experiments, ester hydrolysis did not occur.

After the equilibration process, the organic phase was analyzed by gas chromatography to determine the remaining concentration of the reactant. The concentration difference between the sample and the control solution (initial solution of hexane with reactant) was used to quantify the amount of reactant that had partitioned into the aqueous phase. With the concentration difference obtained and the assumption of constant volumes of the two phases, the concentration of the reactant in the aqueous phase was calculated. The assumption of constant volumes of the two solvent phases was justified since only very low reactant concentrations (mole fraction of the reactants < 0.02) occurred in the experiments. The mutual solubilities of water and hexane are even lower.

Gas Chromatographic Analysis

For the gas chromatographic analysis, 1 µl of the organic phase was injected into a Varian gas chromatograph CP-3800 (USA) equipped with an autosampler and a flame ionization detector. For analysis, the column CP-WAX 58 (FFAP) CB (length 25 m, inner diameter 0.25 mm and 0.2 µm film thickness; Chrompack Inc., USA) and nitrogen as carrier gas (flow 0.5 ml/min) was used. The GC was operated isothermally at 130°C. All samples were analyzed in triplicate. With the use of decane (20 mmol/l) as an internal standard, a five-point calibration curve was acquired each time directly before the analysis of a new series of actual samples.

Model Calculations

Besides the experimental determination of the phase equilibria, the equilibrium compositions of the two phases of system A were also determined theoretically. Therefore, a mathematical model was employed which describes the phase equilibration process. The model consists of two compartments, representing the two phases, with the respective mole balances. In each compartment, the thermodynamic activities of the reactants, the two solvents and the internal standard are calculated with the modified UNIFAC (Dortmund) model (Weidlich and Gmehling, 1987) and the corresponding data set from Gmehling *et al.* (1993). As long as the activities of all the components are unequal in the two compartments, substance quantity is exchanged between the two compartments. The driving force for the exchange of substance is the difference in chemical potentials between the two phases.

The model was implemented in the simulation software package gPROMS (PSEnterprise, UK). For the simulations, the initial compositions of the organic and the aqueous phase were chosen as input parameters. After the simulation reached phase equilibrium, the activity coefficients and the compositions of the two phases in terms of number of moles and molar fractions were obtained. In order to

be able to compare the calculated phase compositions with experimental results, the calculated moles of all components were translated into volumetric molar concentrations [mol/l]. After verifying that excess volumes could be neglected, the volumetric molar concentrations c_i of the components i (in either the aqueous or organic phase) were obtained by the use of the following equation:

$$c_i = \frac{n_i}{V_t} = \frac{n_i}{\sum_j \frac{n_j \cdot M_j}{\rho_j}} \quad i = 1 \ \ldots \ j \tag{1}$$

Here, n_i and n_j represent the calculated moles of component i and of components j, respectively, in the considered phase. V_t characterizes the total volume of the considered phase. M_j denote the molar masses and ρ_j the densities of the pure components j.

RESULTS AND DISCUSSION

Theoretical and experimental analyzes of the equilibrium concentrations of the reactants 1-butanol, propionic acid and butyl propionate in the three two-phase systems with the different aqueous phases were performed. The first goal of this investigation was to test the common assumption of constant partition coefficients and to examine the quality of the UNIFAC predictions. The final goal was to find suitable correlations for the mathematical description of the reactant partitioning behavior.

Ternary Systems: One Reactant Plus Two Solvent Phases

At first, the results of the ternary systems with only one reactant plus the two solvent phases are presented. The partitioning of the compounds 1-butanol and propionic acid was studied with all the three aqueous phases (A, B, C) whereas the partitioning behavior of butyl propionate was only investigated with the two fluid aqueous systems (A, B).

Both experimentally and theoretically (UNIFAC) obtained equilibrium concentrations of the reactants in the two solvent phases are shown in Figs. 1–3. The first conclusion, which can be derived from the experimental results, is that the partitioning behavior of the two polar reactants cannot be described by constant partition coefficients as is commonly done in the field of biocatalysis in aqueous–organic two-phase systems (Antczak *et al*., 2001; Dias *et al*., 1991; Ferreira-Dias and da Fonseca, 1995; Frense *et al*., 1996; Monot *et al*., 1991; Mustranta *et al*., 1993; Suye *et al*., 2002; Wehtje and Adlercreutz, 1997; Wu *et al*., 2002; Yang and Robb, 1994). Compared to the literature, low concentration levels were used in our experiments. However, even at these concentration levels, large errors would occur by the application of constant partition coefficients. The strong non-

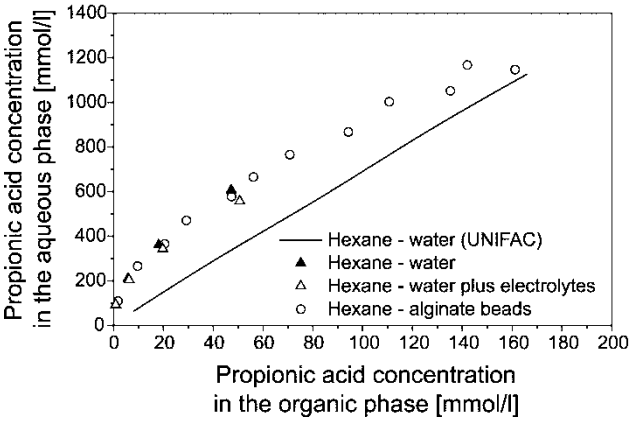

FIGURE 1 Experimental and theoretical phase equilibrium concentrations of propionic acid in aqueous–organic two-phase systems with various compositions of the aqueous phase.

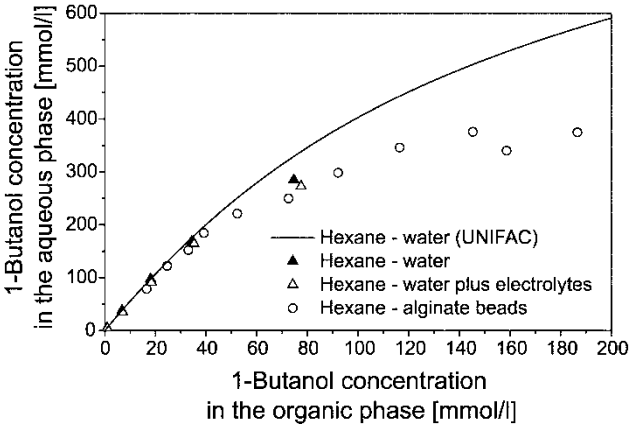

FIGURE 2 Experimental and theoretical phase equilibrium concentrations of 1-butanol in aqueous–organic two-phase systems with various compositions of the aqueous phase.

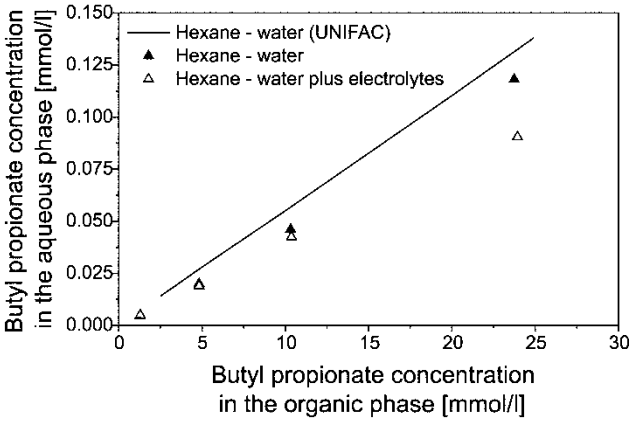

FIGURE 3 Experimental and theoretical phase equilibrium concentrations of butyl propionate in aqueous–organic two-phase systems with various compositions of the aqueous phase.

ideality of the propionic acid partitioning can be explained by a dimerization of acid molecules occurring at higher concentrations in the organic phase (Barcza and Földeáki, 1977). It was found that at propionic acid concentrations in hexane below 1 mmol/l, the acid is almost completely present as monomer while above this concentration the acid primarily exists in dimeric form (Takeda *et al*., 1987). The transition in the propionic acid partitioning behavior, observed around this concentration, is therefore caused by the shift between the monomeric and dimeric acid form.

The comparison of the experimental results obtained from the three different aqueous systems A, B and C reveals that almost no differences in partitioning behavior between the three systems can be identified within the scattering of the experimental values. At least in the concentration range considered here, the presence of electrolytes and alginate polymers included in the aqueous phases B and C hardly changes the partitioning behavior of the considered reactants compared to system A with pure water as aqueous phase. Due to the inability of the pH buffer (present in systems B and C) to counteract the deprotonization of the propionic acid in the aqueous phase, an almost identical pH drop occurred in the propionic acid experiments with the unbuffered (A) and buffered (B, C) systems. For this reason, identical propionic acid partitioning was observed in all three systems.

It can be seen from Figs. 1–3, that the UNIFAC model predicts the correct trend of the equilibrium concentrations. However, large differences (up to 100%) can be observed, for example, between the experimentally and theoretically obtained equilibrium concentrations of propionic acid in the dilute region, which is most likely caused by the fact that the UNIFAC model does not take into account the acid dimerization. Here, the propionic acid equilibrium concentrations in the aqueous phase would be highly underestimated by the UNIFAC model. Due to these partially significant differences between the predicted equilibrium concentrations and the experimentally observed ones, it is obvious that the modified UNIFAC (Dortmund) model, at least in the dilute region applied here, cannot be used to accurately predict the phase equilibria.

From the results presented with the three different aqueous systems it is obvious, that the discrepancies between the modified UNIFAC (Dortmund) model and the experimental values are not caused by the electrolytes present in some of the systems but more likely by the fact that the model is not intended for strongly interacting species and that it still has some deficiencies in predicting infinite-dilution activity coefficients in systems including water (Voutsas and Tassios, 1996; Zhang *et al*., 1998).

Quaternary Systems: Two Reactants Plus Two Solvent Phases

In further investigations, the partitioning behavior of 1-butanol and propionic acid was studied in the case when both reactants were simultaneously present in the system. Experiments were conducted in order to define changes in the equilibrium concentrations of one reactant due to the simultaneous presence of the second one. These experiments were only performed with alginate beads as the aqueous phase (system C).

A comparison of the experimental results of the ternary and quaternary systems can be obtained from Fig. 4 (propionic acid) and Fig. 5 (1-butanol). The results reveal that changes in the partitioning behavior occur when the two considered reactants are present simultaneously. The partitioning behavior of propionic acid is more strongly influenced than that of 1-butanol. Up to a reactant concentration of approximately 10 mmol/l in the organic phase, the partitioning behavior of the reactant can be considered to be independent of the presence of the other reactant. This observation is in good agreement with results of respective simulations with the UNIFAC based model (data not shown).

Empirical Correlations

Our results showed, that the UNIFAC based equilibration model fails to accurately predict the experimentally observed equilibrium concentrations in the systems investigated here. Thus, for a subsequent mathematical modeling of the different processes occurring inside the alginate beads, empirical correlations were derived describing the reactant equilibrium concentrations in the ternary two-phase systems (system C).

Therefore, appropriate functions were fitted to the respective ternary data obtained from the system

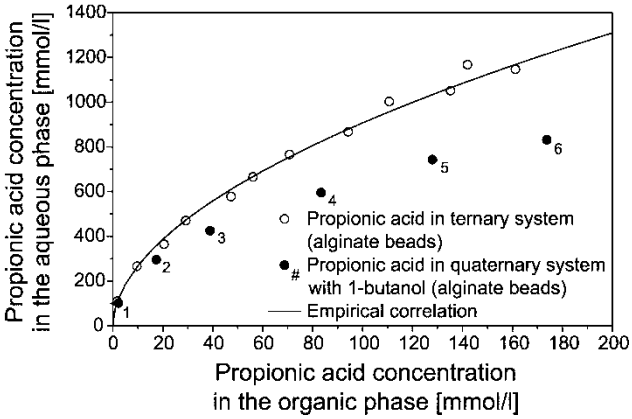

FIGURE 4 Experimental phase equilibrium concentrations of propionic acid in the ternary (only one reactant present) and the quaternary system (two reactants present) with alginate beads as aqueous phase (system C). The indexes next to the solid symbols denote the six investigated quaternary systems. The corresponding 1-butanol equilibrium concentrations are given in Fig. 5.

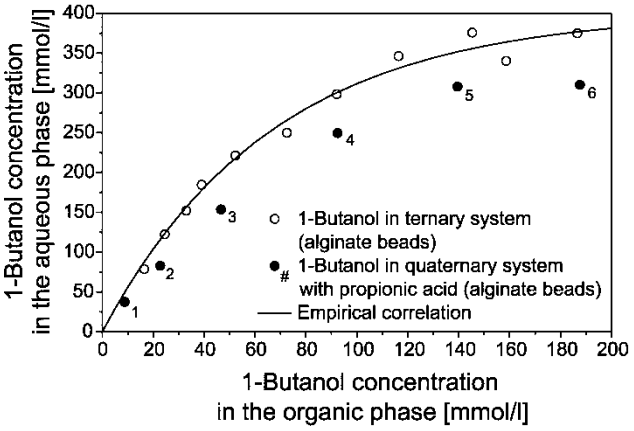

FIGURE 5 Experimental phase equilibrium concentrations of 1-butanol in the ternary (only one reactant present) and the quaternary system (two reactants present) with alginate beads as aqueous phase (system C). The indexes next to the solid symbols denote the six investigated quaternary systems. The corresponding propionic acid equilibrium concentrations are given in Fig. 4.

with the alginate beads. The software Origin (version 5.0; Microcal Software, Inc., USA) was used for the data fitting. The correlations obtained for propionic acid and 1-butanol are given in Table I, while the curves, characterizing the empirical correlations, are shown in Figs. 4 and 5. Also for butyl propionate, the product of the esterification of propionic acid and 1-butanol, a correlation was derived to describe the partitioning behavior between the organic phase and the alginate bead (system C). Therefore, it was assumed that the partitioning in the two-phase system with the alginate beads (C) is the same as in the two experimentally applied fluid aqueous systems (A, B). This was assumed, because the partitioning behavior of butyl propionate could only have been investigated in the systems with the fluid aqueous phases. The assumption was supported by the fact that the other two reactants (1-butanol and propionic acid) partitioned almost equally in all three aqueous systems investigated. The obtained empirical correlation for butyl propionate is given in Table I. Due to the observed dependence of the reactants' partitioning behavior on the presence of another component, in systems with multiple reactants, the derived empirical correlations can strictly only be applied up to concentrations of 10 mmol/l in the organic phase.

TABLE I Empirical correlations describing the equilibrium concentrations of propionic acid, 1-butanol and butyl propionate. The superscript (″) characterizes reactant concentrations in the organic phase whereas (′) represents concentrations in the aqueous phase.

Reactant	Empirical Correlation	a	b
Propionic Acid	$c'_P = a \cdot (c''_P)^b$	79.0 ± 7.3	0.53 ± 0.02
1-Butanol	$c'_B = a \cdot (1 - e^{-b \cdot c''_B})$	401 ± 13	0.015 ± 0.001
Butyl Propionate	$c'_E = a \cdot c''_E$	0.0043 ± 0.0002	–

CONCLUSION

The partitioning behavior of three reactants in different aqueous–organic two-phase systems was studied. Our experimental results revealed that even in the dilute region the partitioning behavior of the polar reactants investigated here cannot be described by constant partition coefficients as is commonly done in the field of biocatalysis in aqueous–organic two-phase systems. Instead of constant partition coefficients, concentration-dependent correlations should be used to describe the non-linear partitioning behavior. Thus, when working with two-phase systems, it is advisable to carefully check the validity of the common assumption of constant partition coefficients to avoid errors in the prediction of equilibrium concentrations. In further experiments it was shown that the electrolytes and polymers, present in the aqueous phase, have hardly an influence on the partitioning behavior of the reactants investigated here. Theoretical calculations with the model developed, based on the modified UNIFAC (Dortmund) method, revealed that this UNIFAC model more or less predicts the general trend of the experimentally observed equilibrium concentrations of the reactants studied. However, in some cases, significant differences between experimentally obtained and predicted values occurred (up to 100%).

In conclusion, it may be said, that, despite the common presence of electrolytes (buffers) in the aqueous phase of two-phase systems used for biocatalysis, the UNIFAC model is still a versatile tool to obtain rough trend estimates of reactant equilibrium concentrations. However, if accurate descriptions of equilibrium concentrations are required (for example for modeling purposes) use of this model is not recommended. Thus, for the system investigated here, empirical correlations were derived to obtain a higher accuracy. Nevertheless, to overcome the restrictions of these correlations, whose application (in terms of concentration range) and transferability is rather limited, semitheoretical models are still required.

Acknowledgements

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